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(54) Title: NOVEL CARBAMYLATED EPO AND METHOD FOR ITS PRODUCTION

(57) Abstract: The present invention comprises an optimal process for carbamylation yielding a product with a low degree of polymerisation and aggregation. The fully carbamylated erythropoietin with all of the N-terminal and all lysine residues was obtained. The resulting fully carbamylated pure EPO is along with pharmaceutical compositions comprising the compound are part of the invention.

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Novel carbamylated EPO and method for its production

Introduction

The present invention is directed to a novel compound, as well as a method of producing said compound. The novel compound, carbamylated erythropoietin, which is characterised by being carbamylated on all the primary amines of lysines and the N-terminal amino acid of the molecule, and in addition this compound is free of carbamylation of the primary amines of other amino acids in the molecule. Furthermore, this novel compound is free of aggregated proteins and polymers, and is suited for use in pharmaceutical compositions for treatment of diseases in for example the central or peripheral nervous system, and other tissues that express the central EPO receptor. One other surprising advantage of the present method of production is the fact that the method provides a product that contains less aggregated protein and fewer polymers, than the products achieved from other known carbamylation methods described for erythropoietin.

Background of the invention

The impairment of biological hematopoietic activity of carbamylated EPO has been shown by Satake, R. et al. (1990) Biochimica et Biophysica Acta; 1038: 125-129 and Mun, K-C. and Golper, T.A. (2000) Blood Purif.; 18: 13-17. Brines et al. 2003, US patent application 20030072737 showed that the loss of the hematopoietic activity did not interfere with the tissue protective properties of EPO.

Some extent of carbamylation of proteins is known as a side effect of using urea in purification of proteins and as a result of high serum urea levels. This is caused by spontaneously decomposition of urea to cyanate at a pH greater than 7 (alkaline conditions). Cyanate is responsible for the carbamylation of the primary amines (α-amino groups and ε-amino groups) of the protein hence the N-terminal end and lysines of a protein are susceptible to carbamylation (Figure 1). Additionally other potential amino acid residues susceptible to carbamylation are e.g. arginine, cysteine, tyrosine, aspartic acid, glutamic acid and histidine; the reaction is however pH dependent and does not proceed as readily as with the N-terminal and lysine residue.

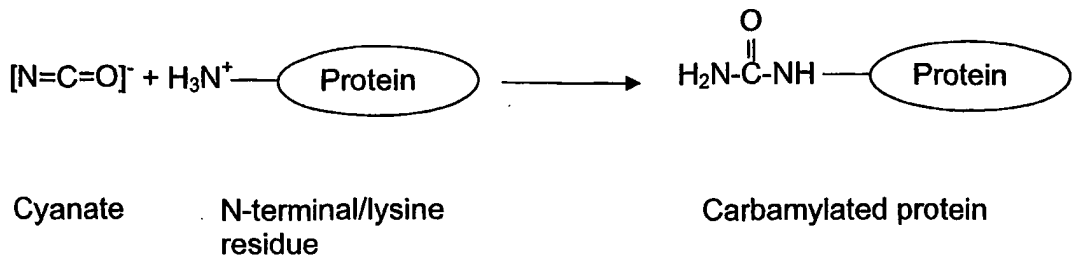

Figure 1. The reaction of cyanate with the N-terminal and lysine residues of proteins.

Investigations to reveal if carbamylation of proteins was able to improve or impair the biological activity of proteins have been conducted by Hörkkö, S. *et al.* (1992) Kidney International.; 41: 1175-1181, Plapp, B.V. et al. (1971) Jour. Biol. Chem.; 246(4): 939-945, Satake, R. et al. (1990) Biochimica et Biophysica Acta; 1038: 125-129 and Mun, K-C. and Golper, T.A. (2000) Blood Purif.; 18: 13-17. They investigated the biological effect of carbamylation of proteins by employing KCNO as source of cyanate. They all observed a decline or change in the biological activity as a result of increased carbamylation. The assessment of degree of carbamylation was based on two analytical methods:

1. Measurement of the decline in free amino groups using a trinitrobenzenesulfonic acid (TNBS) assay and
2. Amino acid analysis determining the lysines converted to homocitrulline residues.

Hörkkö, S. *et al.* (1992), carbamylated a low-density lipoprotein for the maximum of 6 hours at 37° with 2 M KCNO but did not obtained a fully carbamylated protein measured with the TNBS assay.

Plapp, B.V. *et al.* (1971), investigated the effect of time and obtained an almost fully carbamylated bovine pancreatic deoxyribonuclease A after a 24 hour treatment with 1 M KCNO at 37°C.

Mun, K-C. and Golper, T.A. (2000) investigated the effect of time with the maximum of 6 hours reaction time using 2 M KCNO. They also investigated the effect of increasing KCNO concentration at 6 hours all reactions were at 37°C. Mun, K-C. and Golper, T.A. (2000) could not from the experimental design verify the exact degree of carbamylation (please refer to page 16 line 33-35).

We have now in the present invention found that the prior art methods for carbamylation of EPO yielded polymers and aggregates hence making it unsuitable as a biopharmaceutical. In addition we found that the formation of these polymers and aggregates were dependent on the process conditions for the carbamylation. Hence the development of a process was needed with optimal parameters regarding pH, time, cyanate concentration, temperature, protein concentration and most importantly the degree of protein polymerisation. The present invention comprises an optimal process for carbamylation yielding a product with a low degree of polymerisation and aggregation, and furthermore, surprisingly, we have found that a fully carbamylated EPO aiming at the N-terminal and all lysine residues (the latter occurs in a specified pH-range) was obtained. A Subsequent step of the method of the invention was made in order to remove the formed aggregates and polymers. The resulting fully carbamylated pure EPO is a novel compound, and is claimed as such in the present application, along with pharmaceutical compositions comprising the compound.

It has previously been illustrated that the degree of carbamylation depends on cyanate concentration and time. However it has not been described how to obtain a scaleable carbamylation process for production of a biopharmaceutical.

The presence of aggregates by sub-optimal production of erythropoietin has been associated with the induction of antibodies. And the presence of aggregates therefore results in a biopharmaceutical product unsuitable for use in humans.

The carbamylation and purification process described in the present invention leads to a protein that is characterized as fully carbamylated with the lowest formation of polymers or aggregates as possible and with the minimum loss of end product. Hence making it an economical viable step.

Further processing of the carbamylated protein renders a product useful as a biopharmaceutical with only a minimal risk for the generation of an immunological response to the protein due to aggregates and polymers.

The analytical methods for assessment of full carbamylation are in addition to amino acid analysis; TNBS for free primary amino groups and finally a characterization of the product and digested product by MALDI-TOF.

The novel compound of the invention which is erythropoietin that is fully carbamylated on free amino groups at the N-terminal and lysines of the molecule and further is not aggregated and not polymerised to a content of above 2.5 %, and contains a minimum of impurities comprising over

or under carbamylated erythropoietin. It may be used for the production of pharmaceutical compositons for the treatment of diseases responsive to the neuroprotective effects of native erythropoietin.

Summary of the invention

The present invention relates to a scaleable protein carbamylation procedure for the production of biopharmaceuticals. Furthermore, it relates to the product of the process, and to pharmaceutical compositions comprising the compound, and to the use of those compositions.

The carbamylation and purification process described in the present application leads to a protein that is characterized as fully carbamylated with the lowest formation of polymers or aggregates as possible and with the minimum loss of end product.

The carbamylation process has been optimised to yield a fully carbamylated protein with the lowest amount of polymer and aggregates making it an economical viable process. The final product additionally contains limited amounts impurities comprising over and/or under carbamylated erythropoietin (below or above 9 carbamylations per molecule). Further processing of the carbamylated protein removes aggregated and polymerised product to a level of maximum 3% or 2.5 %, and thereby renders a product useful as a biopharmaceutical with only minimal risk of generation of an immunological response to the protein due to aggregates and polymers.

The analytical methods for assessment of full carbamylation are in addition to amino acid analysis; TNBS for free primary amino groups and a characterization of the product and digested product by MALDI-TOF and LC-MS/MS.

Detailed description of the invention

Method

Six steps constitute the method for carbamylation of the proteins:

1. Concentration by ultrafiltration
2. Modification by carbamylation.
3. Desalting by gelfiltration
4. *Purification by anion-exchange.*
5. *Concentration and buffer exchange by ultra- and diafiltration.*
6. *0.22 μm filtration*

The starting material of the present carbamylation process is advantageously purified human EPO, but can be any EPO form of animal or human type, in non-limiting example being it synthetic, recombinant human EPO or biologically or chemically modified human EPO, mutants of human EPO i.e. a molecule where changes in the amino acid sequence are introduced, EPO fragments, peptides of EPO other proteins or a mixture of proteins if several proteins are desired carbamylated.

The first step of the process involves a protein concentration adjustment by ultrafiltration wherein the protein concentration is adjusted for the purpose of keeping a low process volume. The protein concentration is about 0.05-10 mg/ml or about 0.05 –8 mg/ml a preferred embodiment of about 0.05-7 mg/ml most preferably about 2-5 mg/ml.
If the concentration is increased aggregates are increasingly formed. The ultrafiltration is performed by means of a BioMax (Millipore) with a MWCO of 5 kDa. Other filters may be applied. In addition the solubility of the protein may be adjusted by adding stabilizers.

After completion of the concentration step, the protein solution is mixed with potassium borate tetra hydrate, potassium cyanate, with a pH of about 7-11 or pH 7-10 in a preferred embodiment of pH at 8-10 most preferred 9.0 at 0°-60°C or 0°-50°C or 0°-40°C or 0°-< 37°C but at a preferred embodiment a temperature interval of 30-34°C preferably 32°C for a time window of 10 minutes-30 days or 30 minutes-30 days or 1 hour-30 days or 1 hour-20 days or 1 hour-10 days or 1 hour-5 days or 1 hour-2 days or 1 hour-26 hours or 18-26 hours or preferred 22 hours-26 hours most preferably 24 hours. However these preferred intervals could be changed if other process parameters are changed i.e. temperature, cyanate concentration and protein concentration.
If the temperature is below the limits the yield will be low as carbamylation will be slow and inefficient, if the temperature limits are exceeded, the yield will be low due to increased aggregation. Another crucial parameter is time as the carbamylation will not be complete if the time is decreased or if time is increased the formation of aggregates are observed hence resulting in lower yield.
Therefore a process with coherent parameters are presented i.e. if the temperature is lowered the decreased carbamylation reaction can be compensated for by increasing the cyanate concentration and/or reaction time. Additionally, if reaction time is reduced the decreased carbamylation reaction can be compensated for by increasing temperature and/or cyanate concentration. Finally in a process with reduced cyanate concentration the decreased

carbamylation reaction can be compensated for by increasing the reaction time and/or temperature.

Therefore in conclusion one significant change of one crucial parameter (time, temperature, cyanate concentration and protein concentration) would imply a change in one or more of the other crucial parameters in order to obtain a fully carbamylated molecule with low formation of aggregates and polymers.

The concentration of borate buffer may be 0.05-2 M but in a preferred embodiment 0.1-1 M and most preferably 0.5 M as cyanate inherently hydrolyses and polymerizes under uptake of protons and lack of buffer capacity results in a drift of the pH of the solution. A concentration of 0.5 M borate buffer is required to control the pH drift caused by proton uptake of the 0.5 M cyanate concentration in use. Additionally other reaction buffers than borate may be employed e.g. a carbonate buffer or phosphate buffer.

In addition the cyanate concentration is preferred in the range of about 0.05-10 M or about 0.05-8 M or about 0.05-6 M or about 0.05-4 M or about 0.05-2 M a preferred embodiment of about 0.05-1 M most preferably about 0.5 M. A process using other salts of cyanate and borate may be employed.

The desalting and buffer change of proteins can be performed by dialysis, dia-ultrafiltration or by means of a chromatographic gelfiltration. The desalting of the reaction mixture of protein and cyanate is performed by means of a chromatographic gelfiltration. The G-25 Fine (Amersham Biosciences) matrix is employed. Other gelfiltration matrices may be applied such as for example matrices of crosslinked polysaccharides or crosslinked mixed polysaccharides, polyacrylamide, polystyrene or matrices of a ceramic nature. Furthermore the column height may be varied in this step. The hold up time before sample application to the column is controlled and should not exceed 2 hours, as this would cause further carbamylation and polymer formation.

The carbamylation step may be adjusted to obtain a product with less than 30 % aggregates and polymers or less than 25% or less than 20 % or less than 15 % or less than 12.5 % or less than 10% or less than 8% or less than 7%.

The removal of aggregates and polymers is perfomed by a purification step using anionexchange. It is observed that it can separate carbamylated EPO from remains of the starting material and from aggregates/polymers.

The running buffer A is: 0.3% Tris (25 mM), 0.3% NaCl (50 mM). pH 8.5 ± 0.2, and elution buffer B: 0.3% Tris (25 mM), 5.8% (1 M) NaCl. pH 8.5 ± 0.2

The gradient is performed with 0-30 % over 20 column volumes yielding the desired separation. The purification step may result in a product with less than 3% aggregates and polymers or less than 2.5% or less than 2% or less than 1.5% or less than 1% or less than 0.5%.

The elution mode, collection and pooling of the carbamylated-EPO peak influence the distribution of the hetereogenity of the eluted protein. For example, the amounts of impurities comprising over and under carbamylated forms of EPO will vary depending on the collection and pooling procedure. Thereby, a composition comprising fully carbamylated EPO and impurities with over or under carbamylated EPO to an extent that amounts to less than 40 % w/w of total carbamylated EPO, or less than 35% or less than 30% or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5 % or less than 4 % or less than 3 % or less than 2 % or less than 1 %.

In addition to influencing the overall content of impurities, the collection and pooling of the carbamylated EPO peak, influence the distribution between under and over carbamylated EPO, so that the amount of over carbamylated EPO may be less than 35 % w/w of the total carbamylated EPO or less than 32.5 % or less than 30 % or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5 % or less than 4 % or less than 3 % or less than 2 % or less than 1 %.

Other running and elution buffers may be employed as other anionexchange matrices and charged filters may be employed. The matrices in unlimiting example being of crosslinked polysaccharides or crosslinked mixed polysaccharides, polyacrylamide, polystyrene or matrices of ceramic nature.

In addition even cation exchange, hydrophobic interaction chromatography, reversed phase chromatography, affinity chromatography and size exclusion chromatography may be used for the purification.

Next step for the adjustment of concentration and buffer a dia/ultrafiltration tangential flow filtration unit is used. The carbamylated EPO is adjusted to a concentration > 0.5 mg/ml and the buffer changed to a 20 mM citrate, 100 mM NaCl buffer.

The concentration and buffer change is performed by means of a BioMax (Millipore) with a MWCO of 5 kDa. Other filters may be employed.

Finally the purified biopharmacutical drug substance is 0.22 µm filtrated using a Millipak (Millipore) to reduce germs. Any 0.22 µm filter may be used.

Using the method a fully carbamylated EPO is obtained with less than 3 % or preferably less than 2.5% of aggregates as measured by SEC-HPLC. The full carbamylation of the 8 lysine residues is verified using aminoacid analysis determining the converted lysines to homocitrulline. Furthermore the carbamylation was followed using the TNBS assay for determination of primary amines hence showing the complete carbamylation of lysines and the N-terminal.

In addition a thorough characterization using MALDI-TOF for the determination of the change in the intact mass both of the PNGase treated protein and for the protein with the N-glycans. In addition MALDI-TOF peptide mass fingerprint analysis/LC-MS/MS analysis. Thus measuring the modification of EPO and showing that all 8 lysines and the N-terminal is carbamylated. No other carbamylated aminoacids were detected and no modifications of the glycans were detected. Furthermore, a reduced content of impurities (over and under carbamylated forms of EPO) is obtained in the final product. This product is novel and claimed.

One embodiment of the invention is the composition obtained after the carbamylation step, but before the anion exchange purification, comprising fully carbamylated EPO and with less than 30 % w/w aggregates and polymers or less than 25% or less than 20 % or less than 15 % or less than 12.5 % or less than 10% or less than 8% or less than 7%.

One further embodiment of the invention is the composition obtained after the anion exchange purification comprise fully carbamylated EPO with less than 3% w/w aggregates and polymers or less than 2.5% or less than 2% or less than 1.5% or less than 1% or less than 0.5%. Further, this composition comprise impurities consisting of over- or under- carbamylated EPO to an extent that amounts to less than 40 % w/w of total carbamylated EPO, or less than 35% or less than 30% or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5

% or less than 4 % or less than 3 % or less than 2 % or less than 1 %. Further, the amount of over carbamylated EPO in the composition may be less than 35 % w/w of the total carbamylated EPO or less than 32.5 % or less than 30 % or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5 % or less than 4 % or less than 3 % or less than 2 % or less than 1 %.

PHARMACEUTICAL COMPOSITIONS OF THE INVENTION

One aspect of the invention is the use of the compounds of the invention for the production of pharmaceutical compositions to be used in humans or mammals for treatment of the conditions described below.

One embodiment of the invention is a pharmaceutical composition comprising a therapeutically effective amount of fully carbamylated EPO, less than 3 % w/w aggregates/polymers or less than 2.5% or less than 2% or less than 1.5% or less than 1% or less than 0.5% and further, this composition comprise impurities consisting of over- or under- carbamylated EPO to an extent that amounts to less than 40 % w/w of total carbamylated EPO, or less than 35% or less than 30% or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5 % or less than 4 % or less than 3 % or less than 2 % or less than 1 %. Further, the amount of over-carbamylated EPO in the composition may be less than 35 % w/w of the total carbamylated EPO or less than 32.5 % or less than 30 % or less than 27.5% or less than 25% or less than 22.5% or less than 20 % or less than 17.5 % or less than 15 % or less than 12.5 % or less than 10 % or less than 7.5 % or less than 5 % or less than 4 % or less than 3 % or less than 2 % or less than 1 % .In the practice of one aspect of the present invention, a pharmaceutical composition as described above containing the compound of the invention may be administerable to a mammal by any route that provides a sufficient level of the compound of the invention in the vasculature to permit translocation across an endothelial cell barrier and beneficial effects on responsive cells. When used for the purpose of perfusing a tissue or organ, similar results are desired. In the instance where the cells or tissue is non-vascularized and/or the administration is by bathing the cells or tissue with the composition of the invention, the pharmaceutical composition provides an effective responsive cell-beneficial amount of a compound of the invention. The endothelial cell barriers across which the compound of the invention may translocate include

tight junctions, perforated junctions, fenestrated junctions, and any other types of endothelial barriers present in a mammal. A preferred barrier is an endothelial cell tight junction, but the invention is not so limiting.

The aforementioned compound of the invention is useful generally for the therapeutic or prophylactic treatment of human diseases of the central nervous system or peripheral nervous system which have primarily neurological or psychiatric symptoms, ophthalmic diseases, cardiovascular diseases, cardiopulmonary diseases, respiratory diseases, kidney, urinary and reproductive diseases, gastrointestinal diseases and endocrine and metabolic abnormalities. In particular, such conditions and diseases include hypoxic conditions, which adversely affect excitable tissues, such as excitable tissues in the central nervous system tissue, peripheral nervous system tissue, or cardiac or retinal tissue such as, for example, brain, heart, or retina/eye. Therefore, the compound of the invention can be used to treat or prevent damage to excitable tissue resulting from hypoxic conditions in a variety of conditions and circumstances. Non-limiting examples of such conditions and circumstances are provided in the table hereinbelow.

In the example of the protection of neuronal tissue pathologies treatable in accordance with the present invention, such pathologies include those resulting from reduced oxygenation of neuronal tissues. Any condition which reduces the availability of oxygen to neuronal tissue, resulting in stress, damage, and finally, neuronal cell death, can be treated by the methods of the present invention. Generally referred to as hypoxia and/or ischemia, these conditions arise from or include, but are not limited to, stroke, vascular occlusion, prenatal or postnatal oxygen deprivation, suffocation, choking, near drowning, carbon monoxide poisoning, smoke inhalation, trauma, including surgery and radiotherapy, asphyxia, epilepsy, hypoglycemia, chronic obstructive pulmonary disease, emphysema, adult respiratory distress syndrome, hypotensive shock, septic shock, anaphylactic shock, insulin shock, sickle cell crisis, cardiac arrest, dysrhythmia, nitrogen narcosis, and neurological deficits caused by heart-lung bypass procedures.

In one embodiment, for example, the specific pharmaceutical compositions comprising the composition of the invention can be administered to prevent injury or tissue damage resulting from risk of injury or tissue damage during surgical procedures, such as, for example, tumor resection or aneurysm repair. Other pathologies caused by or resulting from hypoglycemia which are treatable by the methods described herein include insulin overdose,

also referred to as iatrogenic hyperinsulinemia, insulinoma, growth hormone deficiency, hypocortisolism, drug overdose, and certain tumors.

Other pathologies resulting from excitable neuronal tissue damage include seizure disorders, such as epilepsy, convulsions, or chronic seizure disorders. Other treatable conditions and diseases include, but are not limited to, diseases such as stroke (Ischemic stroke, subarachnoid haemorrhage, Intracerebral haemorrhage), multiple sclerosis, hypotension, cardiac arrest, Alzheimer's disease, Parkinson's disease, cerebral palsy, brain or spinal cord trauma, AIDS dementia, age-related loss of cognitive function, memory loss, amyotrophic lateral sclerosis, seizure disorders, alcoholism, retinal ischemia, optic nerve damage resulting from glaucoma, and neuronal loss.

The specific composition and methods of the present invention may be used to treat inflammation resulting from disease conditions or various traumas, such as physically or chemically induced inflammation. Such traumas could include angitis, chronic bronchitis, pancreatitis, osteomyelitis, rheumatoid arthritis, glomerulonephritis, optic neuritis, temporal arteritis, encephalitis, meningitis, transverse myelitis, dermatomyositis, polymyositis, necrotizing fascilitis, hepatitis, and necrotizing enterocolitis.

Evidence has demonstrated that activated astrocytes can exert a cytotoxic role towards neurons by producing neurotoxins. Nitric oxide, reactive oxygen species, and cytokines are released from glial cells in response to cerebral ischemia (see Becker, K.J. 2001. Targeting the central nervous system inflammatory response in ischemic stroke. Curr Opinion Neurol 14:349-353 and Mattson, M.P., Culmsee, C., and Yu, Z.F. 2000. Apoptotic and Antiapoptotic mechanisms in stroke. Cell Tissue Res 301:173-187.). Studies have further demonstrated that in models of neurodegeneration, glial activation and subsequent production of inflammatory cytokines depends upon primary neuronal damage (see Viviani, B., Corsini, E., Galli, C.L., Padovani, A., Ciusani, E., and Marinovich, M. 2000. Dying neural cells activate glia through the release of a protease product. Glia 32:84-90 and Rabuffetti, M., Scioratti, C., Tarozzo, G., Clementi, E., Manfredi, A.A., and Beltramo, M. 2000. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone includes long lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. J Neurosci 20:4398-4404). Inflammation and glial activation is common to different forms of neuro degenerative disorders, including cerebral ischemia, brain trauma and experimental allergic encephalomyelitis, disorders in which erythropoietin exerts a neuroprotective effect. Inhibition

of cytokine production by erythropoietin could, at least in part, mediate its protective effect. However, unlike "classical" anti-inflammatory cytokines such as IL-10 and IL-13, which inhibit tumor necrosis factor production directly, erythropoietin appears to be active only in the presence of neuronal death.

While not wishing to be bound by any particular theory, it appears that this anti-inflammatory activity may be hypothetically explained by several non-limiting theories. First, since erythropoietin prevents apoptosis, inflammatory events triggered by apoptosis would be prevented. Additionally, erythropoietin may prevent the release of molecular signals from dying neurons which stimulate the glia cells or could act directly on the glial cells reducing their reaction to these products. Another possibility is that erythropoietin targets more proximal members of the inflammatory cascade (*e.g.,* caspase 1, reactive oxygen or nitrogen intermediates) that trigger both apoptosis and inflammation.

Furthermore, erythropoietin appears to provide anti-inflammatory protection without the rebound affect typically associated with other anti-inflammatory compounds such as dexamethasone. Once again, not wishing to be bound by any particular theory, it appears as though this may be due to erythropoietin's affect on multipurpose neuro toxins such as nitric oxide (NO). Although activated astrocytes and microglia produce neurotoxic quantities of NO in response to various traumas, NO serves many purposes within the body including the modulation of essential physiological functions. Thus, although the use of an anti-inflammatory may alleviate inflammation by suppressing NO or other neuro toxins, if the anti-inflammatory has too long a half-life it may also interfere with these chemicals' roles in repairing the damage resulting from the trauma that led to the inflammation. It is hypothesized that the compound of the present invention is able to alleviate the inflammation without interfering with the restorative capabilities of neurotoxins such as NO.

The specific compositions and methods of the invention may be used to treat conditions of, and damage to, retinal tissue. Such disorders include, but are not limited to retinal ischemia, macular degeneration, retinal detachment, retinitis pigmentosa, arteriosclerotic retinopathy, hypertensive retinopathy, retinal artery blockage, retinal vein blockage, hypotension, and diabetic retinopathy.

In another embodiment, the methods and principles of the invention may be used to protect or treat injury resulting from radiation damage or chemotherapy induced damage to excitable tissue. A further utility of the methods of the present invention is in the treatment of

neurotoxin poisoning, such as domoic acid shellfish poisoning, neurolathyrism, and Guam disease, amyotrophic lateral sclerosis, and Parkinson's disease.

As mentioned above, the present invention is also directed to a method for enhancing excitable tissue function in a mammal by peripheral administration of a compound of the invention as described above. Various diseases and conditions are amenable to treatment using this method, and further, this method is useful for enhancing cognitive function in the absence of any condition or disease. These uses of the present invention are described in further detail below and include enhancement of learning and training in both human and non-human mammals.

Conditions and diseases treatable by the methods of this aspect of the present invention directed to the central nervous system include, but are not limited to, mood disorders, anxiety disorders, depression, autism, attention deficit hyperactivity disorder, and cognitive dysfunction. These conditions benefit from enhancement of neuronal function. Other disorders treatable in accordance with the teachings of the present invention include for example, sleep disruption, sleep apnea, and travel-related disorders; subarachnoid and aneurismal bleeds, hypotensive shock, concussive injury, septic shock, anaphylactic shock, and sequelae of various encephalitides and meningitides, for example, connective tissue disease-related cerebritides such as lupus. Other uses include prevention of or protection from poisoning by neurotoxins, such as domoic acid shellfish poisoning, neurolathyrism, and Guam disease, amyotrophic lateral sclerosis, Parkinson's disease; postoperative treatment for embolic or ischemic injury; whole brain irradiation; sickle cell crisis; and eclampsia.

A further group of conditions treatable by the methods of the present invention include mitochondrial dysfunction, of either a hereditary or an acquired nature, which are the cause of a variety of neurological diseases typified by neuronal injury and death. For example, Leigh disease (subacute necrotizing encephalopathy) is characterized by progressive visual loss and encephalopathy, due to neuronal drop out, and myopathy. In these cases, defective mitochondrial metabolism fails to supply enough high energy substrates to fuel the metabolism of excitable cells. An erythropoietin receptor activity modulator optimizes failing function in a variety of mitochondrial diseases. As mentioned above, hypoxic conditions adversely affect excitable tissues. The excitable tissues include, but are not limited to, central nervous system tissue, peripheral nervous system tissue, and heart tissue. In addition to the conditions described above, the methods of the present invention are useful in the treatment of inhalation poisoning,

such as carbon monoxide and smoke inhalation, severe asthma, adult respiratory distress syndrome, choking, and near drowning. Further conditions which create hypoxic conditions or by other means induce excitable tissue damage include hypoglycemia that may occur in inappropriate dosing of insulin, or with insulin-producing neoplasms (insulinoma).

Various neuropsychologic disorders which are believed to originate from excitable tissue damage are treatable by the instant methods. Chronic disorders in which neuronal damage is involved and for which treatment by the present invention is provided include disorders relating to the central nervous system and/or peripheral nervous system including age-related loss of cognitive function and senile dementia, chronic seizure disorders, Alzheimer's disease, Parkinson's disease, dementia, memory loss, amyotrophic lateral sclerosis, multiple sclerosis, tuberous sclerosis, Wilson's disease, cerebral and progressive *supra*nuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease, Huntington's disease, myotonic dystrophy, Charcot-Marie-Tooth Disease, Friedrich's ataxia and other ataxias, as well as Gilles de la Tourette's syndrome, seizure disorders such as epilepsy and chronic seizure disorder, stroke, brain or spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to, schizophrenia, schizoaffective disorder, attention deficit disorder hyperactivity, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM), the most current version, IV, of which in incorporated herein by reference in its entirety.

In another embodiment, recombinant chimeric toxin molecules comprising a compound of the invention can be used for therapeutic delivery of toxins to treat a proliferative disorder, such as cancer, or viral disorder, such as subacute sclerosing panencephalitis.

The following table lists additional exemplary, non-limiting indications as to the various conditions and diseases amenable to treatment by the aforementioned compounds of the invention.

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
Heart	Ischemia	Coronary artery disease	Acute, chronic Stable, unstable

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
		Myocardial infarction	Dressler's syndrome
		Angina	
		Congenital heart disease	Valvular Cardiomyopathy
		Prinzmetal angina	
		Cardiac rupture	Aneurysmatic Septal perforation
		Angiitis	
	Arrhythmia	Tachy-, bradyarrhythmia *Supra*ventricular, ventricular Conduction abnormalities	Stable, unstable Hypersensitive carotid sinus node
	Congestive heart failure	Left, right, bi-ventricular, systolic, diastolic	Cardiomyopathies, such as idiopathic familial, infective, metabolic, storage disease, deficiencies, connective tissue disorder, infiltration and granulomas, neurovascular
		Myocarditis	Autoimmune, infective, idiopathic
		Cor pulmonale	
	Blunt and penetrating trauma		
	Toxins	Cocaine toxicity	
Vascular	Hypertension	Primary, secondary	
	Decompression sickness		
	Fibromuscular hyperplasia		
	Aneurysm	Dissecting, ruptured, enlarging	
Lungs	Obstructive	Asthma Chronic bronchitis, Emphysema and airway obstruction	
	Ischemic lung disease	Pulmonary embolism, Pulmonary thrombosis, Fat embolism	
	Environmental lung diseases		
	Ischemic lung disease	Pulmonary embolism Pulmonary thrombosis	
	Interstitial lung disease	Idiopathic pulmonary fibrosis	
	Congenital	Cystic fibrosis	
	Cor pulmonale		
	Trauma		
	Pneumonia and pneumonitides	Infectious, parasitic, toxic, traumatic, burn, aspiration	
	Sarcoidosis		
Pancreas	Endocrine	Diabetes mellitus, type I and II	Beta cell failure, dysfunction Diabetic neuropathy
		Other endocrine cell failure of the pancreas	
	Exocrine	Exocrine pancreas failure	pancreatitis

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
Bone	Osteopenia	Primary secondary	Hypogonadism immobilisation Postmenopausal Age-related Hyperparathyroidism Hyperthyroidism Calcium, magnesium, phosphorus and/or vitamin D deficiency
	Osteomyelitis		
	Avascular necrosis		
	Trauma		
	Paget's disease		
Skin	Alopecia	Areata Totalis	Primary Secondary Male pattern baldness
	Vitiligo	Localized generalized	Primary secondary
	Diabetic ulceration		
	Peripheral vascular disease		
	Burn injuries		
Autoimmune disorders	Lupus erythematodes, Sjiogren, Rheumatoid arthritis, Glomerulonephritis, Angiitis		
	Langerhan's histiocytosis		
Eye	Optic neuritis		
	Blunt and penetrating injuries, Infections, Sarcoid, Sickle C disease, Retinal detachment, Temporal arteritis		
	Retinal ischemia, Macular degeneration, Retinitis pigmentosa, Arteriosclerotic retinopathy, Hypertensive retinopathy, Retinal artery blockage, Retinal vein blockage, Hypotension, Diabetic retinopathy, and Macular edema		
Embryonic and fetal disorders	Asphyxia		
	Ischemia		
CNS	Chronic fatigue syndrome, acute and chronic hypoosmolar and hyperosmolar syndromes, AIDS Dementia, Electrocution		
	Encephalitis	Rabies, Herpes	
	Meningitis		
	Subdural hematoma		
	Nicotine addiction		

Cell, tissue or organ	*Dysfunction or pathology*	*Condition or disease*	*Type*
	Drug abuse and withdrawal	Cocaine, heroin, crack, marijuana, LSD, PCP, poly-drug abuse, ecstasy, opioids, sedative hypnotics, amphetamines, caffeine	
	Obsessive-compulsive disorders		
	Spinal stenosis, Transverse myelitis, Guillian Barre, Trauma, Nerve root compression, Tumoral compression, Heat stroke		
ENT	Tinnitus Meuniere's syndrome Hearing loss		
	Traumatic injury, barotraumas		
Kidney	Renal failure	Acute, chronic	Vascular/ischemic, interstitial disease, diabetic kidney disease, nephrotic syndromes, infections, injury, contrast-induced, chemotherapy-induced, CPB-induced, or preventive
	Henoch S. Purpura		
Striated muscle	Autoimmune disorders	Myasthenia gravis Dermatomyositis Polymyositis	
	Myopathies	Inherited metabolic, endocrine and toxic	
	Heat stroke		
	Crush injury		
	Rhabdomylosis		
	Mitochondrial disease		
	Infection	Necrotizing fasciitis	
Sexual dysfunction	Central and peripheral (e.g. erectile dysfunction)	Impotence secondary to medication, (diabetes)	
Liver	Hepatitis	Viral, bacterial, parasitic	
	Ischemic disease		
	Cirrhosis, fatty liver		
	Infiltrative/metabolic diseases		
Gastrointestinal	Ischemic bowel disease		
	Inflammatory bowel disease		
	Necrotizing enterocolitis		
Organ transplantation	Treatment of donor and recipient		
Reproductive tract	Infertility	Vascular Autoimmune Uterine abnormalities Implantation disorders	
Endocrine	Glandular hyper- and hypofunction		

As mentioned above, these diseases, disorders or conditions are merely illustrative of the range of benefits provided by the compound of the invention. Accordingly, this invention generally provides therapeutic or prophylactic treatment of the consequences of mechanical trauma or of human diseases. Therapeutic or prophylactic treatment for diseases, disorders or conditions of the CNS and/or peripheral nervous system are preferred. Therapeutic or prophylactic treatment for diseases, disorders or conditions which have a psychiatric component is provided. Therapeutic or prophylactic treatment for diseases, disorders or conditions including, but not limited to, those having an ophthalmic, cardiovascular, cardiopulmonary, respiratory, kidney, urinary, reproductive, gastrointestinal, endocrine, or metabolic component is provided.

In one embodiment, such a pharmaceutical composition comprising the compound of the invention may be administered systemically to protect or enhance the target cells, tissue, or organ. Such administration may be parenterally, via inhalation, or transmucosally, *e.g.*, orally, nasally, rectally, intravaginally, sublingually, submucosally or transdermally. Preferably, administration is parenteral, *e.g.*, via intravenous or intraperitoneal injection, and also including, but is not limited to, intra-arterial, intramuscular, intradermal and subcutaneous administration.

For other routes of administration, such as by use of a perfusate, injection into an organ, or other local administration, a pharmaceutical composition will be provided which results in similar levels of the compound of the invention as described above. A level of about 0.01pM – 30 nM is preferred.

The pharmaceutical compositions of the invention may comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized foreign pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc,

sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

Pharmaceutical compositions adapted for oral administration may be provided as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions. Tablets or hard gelatine capsules may comprise lactose, starch or derivatives thereof, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, stearic acid or salts thereof. Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc. Solutions and syrups may comprise water, polyols, and sugars.

An active agent intended for oral administration may be coated with or admixed with a material that delays disintegration and/or absorption of the active agent in the gastrointestinal tract (*e.g.*, glyceryl monostearate or glyceryl distearate may be used). Thus, the sustained release of an active agent may be achieved over many hours and, if necessary, the active agent can be protected from being degraded within the stomach. Pharmaceutical compositions for oral administration may be formulated to facilitate release of an active agent at a particular gastrointestinal location due to specific pH or enzymatic conditions.

Pharmaceutical compositions adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Pharmaceutical compositions adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays,

aerosols or oils. For topical administration to the skin, mouth, eye or other external tissues a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops. In these compositions, the active ingredient can be dissolved or suspended in a suitable carrier, *e.g.*, in an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles, and mouthwashes.

Pharmaceutical compositions adapted for nasal and pulmonary administration may comprise solid carriers such as powders (preferably having a particle size in the range of 20 to 500 microns). Powders can be administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nose from a container of powder held close to the nose. Alternatively, compositions adopted for nasal administration may comprise liquid carriers, *e.g.*, nasal sprays or nasal drops. Alternatively, inhalation directly into the lungs may be accomplished by inhalation deeply or installation through a mouthpiece into the oropharynx. These compositions may comprise aqueous or oil solutions of the active ingredient. Compositions for administration by inhalation may be supplied in specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient. In a preferred embodiment, pharmaceutical compositions of the invention are administered into the nasal cavity directly or into the lungs via the nasal cavity or oropharynx.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of an intended recipient. Other components that may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, *e.g.*, sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile

powders, granules, and tablets. In one embodiment, an autoinjector comprising an injectable solution of a compound of the invention may be provided for emergency use by ambulances, emergency rooms, and battlefield situations, and even for self-administration in a domestic setting, particularly where the possibility of traumatic amputation may occur, such as by imprudent use of a lawn mower. The likelihood that cells and tissues in a severed foot or toe will survive after reattachment may be increased by administering a compound of the invention to multiple sites in the severed part as soon as practicable, even before the arrival of medical personnel on site, or arrival of the afflicted individual with severed toe at the emergency room.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile saline can be provided so that the ingredients may be mixed prior to administration.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

A perfusate composition may be provided for use in transplanted organ baths, for *in situ* perfusion, or for administration to the vasculature of an organ donor prior to organ harvesting. Such pharmaceutical compositions may comprise levels of the compound of the invention not suitable for acute or chronic, local or systemic administration to an individual, but will serve the functions intended herein in a cadaver, organ bath, organ perfusate, or *in situ* perfusate prior to removing or reducing the levels of the compound of the invention contained therein before exposing or returning the treated organ or tissue to regular circulation.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or

biological products, which notice reflects approval by the agency of manufacture, use, or sale for human administration.

In another embodiment, for example, the compound of the invention can be delivered in a controlled-release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); WO 91/04014; U.S. Patent No. 4,704,355; Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Press: Boca Raton, Florida, 1974; Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61, 1953; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the target cells, tissue or organ, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, pp. 115-138 in Medical Applications of Controlled Release, vol. 2, *supra*, 1984). Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In another embodiment, the compound of the invention, as properly formulated, can be administered by nasal, oral, rectal, vaginal, or sublingual administration.

In a specific embodiment, it may be desirable to administer the compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as silastic membranes, or fibers.

Selection of the preferred effective dose will be determined by a skilled artisan based upon considering several factors which will be known to one of ordinary skill in the art. Such

factors include the particular form of compound of the invention, and its pharmacokinetic parameters such as bioavailability, metabolism, half-life, etc., which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and each patient's circumstances, *e.g.*, depending upon the condition and the immune status of the individual patient, and according to standard clinical techniques.

In another aspect of the invention, a perfusate or perfusion solution is provided for perfusion and storage of organs for transplant, the perfusion solution including an amount of the compound of the invention, effective to protect responsive cells and associated cells, tissues, or organs. Transplant includes, but is not limited to, xenotransplantation, where a organ (including cells, tissue or other bodily part) is harvested from one donor and transplanted into a different recipient; and autotransplant, where the organ is taken from one part of a body and replaced at another, including bench surgical procedures, in which an organ may be removed, and while *ex vivo*, resected, repaired, or otherwise manipulated, such as for tumor removal, and then returned to the original location. In one embodiment, the perfusion solution is the University of Wisconsin (UW) solution (U.S. Patent No. 4,798,824) which contains from about 1 to about 25 U/ml erythropoietin, 5% hydroxyethyl starch (having a molecular weight of from about 200,000 to about 300,000 and substantially free of ethylene glycol, ethylene chlorohydrin, sodium chloride and acetone); 25mM KH2PO4; 3mM glutathione; 5mM adenosine; 10mM glucose; 10mM HEPES buffer; 5mM magnesium gluconate; 1.5mM CaCl2; 105mM sodium gluconate; 200,000 units penicillin; 40 units insulin; 16mg Dexamethasone; 12mg Phenol Red; and has a pH of 7.4-7.5 and an osmolality of about 320 mOSm/l. The solution is used to maintain cadaveric kidneys and pancreases prior to transplant. Using the solution, preservation can be extended beyond the 30-hour limit recommended for cadaveric kidney preservation. This particular perfusate is merely illustrative of a number of such solutions that can be adapted for the present use by inclusion of an effective amount of the compound of the invention. In a further embodiment, the perfusate solution contains from about 0.01pg/ml to about 400 ng/ml of the compound of the invention, or from about 40 to about 300 ng/ml of the compound of the invention.

While the preferred recipient of a compound of the invention for the purposes herein throughout is a human, the methods herein apply equally to other mammals, particularly domesticated animals, livestock, companion and zoo animals. However, the invention is not so limiting and the benefits can be applied to any mammal.

THERAPEUTIC AND PREVENTATIVE USES OF THE COMPOUNDS OF THE INVENTION

As noted in Example 1 below, the presence of erythropoietin receptors in the brain capillary human endothelium indicates that the targets of the compounds of the invention are present in the human brain, and that the animal studies on these compounds of the invention are directly translatable to the treatment or prophylaxis of human beings.

In another aspect of the invention, methods and compositions for enhancing the viability of cells, tissues, or organs which are not isolated from the vasculature by an endothelial cell barrier are provided by exposing the cells, tissue or organs directly to a pharmaceutical composition comprising a compound of the invention, or administering or contacting a compound of the invention-containing pharmaceutical composition to the vasculature of the tissue or organ. Enhanced activity of responsive cells in the treated tissue or organ is responsible for the positive effects exerted.

As described above, the invention is based, in part, on the discovery that erythropoietin molecules can be transported from the luminal surface to the basement membrane surface of endothelial cells of the capillaries of organs with endothelial cell tight junctions, including, for example, the brain, retina, and testis. Thus, responsive cells across the barrier are susceptible targets for the beneficial effects of a compound of the invention, and others cell types or tissues or organs that contain and depend in whole or in part on responsive cells therein are targets for the methods of the invention. While not wishing to be bound by any particular theory, after transcytosis of a compound of the invention, the compound of the invention can interact with an erythropoietin receptor on an responsive cell, for example, neuronal, retinal, muscle, heart, lung, liver, kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, pancreas, bone, skin, or endometrial cell, and receptor binding can initiate a signal transduction cascade resulting in the activation of a gene expression program within the responsive cell or tissue, resulting in the protection of the cell or tissue, or organ, from damage, such as by toxins, chemotherapeutic agents, radiation therapy, hypoxia, etc. Thus, methods for

protecting responsive cell-containing tissue from injury or hypoxic stress, and enhancing the function of such tissue are described in detail herein below. As noted above, the methods of the invention are equally applicable to humans as well as to other animals.

In the practice of one embodiment of the invention, a mammalian patient is undergoing systemic chemotherapy for cancer treatment, including radiation therapy, which commonly has adverse effects such as nerve, lung, heart, ovarian, or testicular damage. Administration of a pharmaceutical composition comprising a compound of the invention as described above is performed prior to and during chemotherapy and/or radiation therapy, to protect various tissues and organs from damage by the chemotherapeutic agent, such as to protect the testes. Treatment may be continued until circulating levels of the chemotherapeutic agent have fallen below a level of potential danger to the mammalian body.

In the practice of another embodiment of the invention, various organs were planned to be harvested from a victim of an automobile accident for transplant into a number of recipients, some of which required transport for an extended distance and period of time. Prior to organ harvesting, the victim was infused with a pharmaceutical composition comprising a compound of the invention as described herein. Harvested organs for shipment were perfused with a perfusate containing a compound of the invention as described herein, and stored in a bath comprising a compound of the invention. Certain organs were continuously perfused with a pulsatile perfusion device, utilizing a perfusate containing a compound of the invention in accordance with the present invention. Minimal deterioration of organ function occurred during the transport and upon implant and reperfusion of the organs *in situ*.

In another embodiment of the invention, a surgical procedure to repair a heart valve required temporary cardioplegia and arterial occlusion. Prior to surgery, the patient was infused with 4 μg of a compound of the invention per kg body weight. Such treatment prevented hypoxic ischemic cellular damage, particularly after reperfusion.

In another embodiment of the invention, in any surgical procedure, such as in cardiopulmonary bypass surgery, a compound of the invention can be used. In one embodiment, administration of a pharmaceutical composition comprising a compound of the invention as described above is performed prior to, during, and/or following the bypass procedure, to protect the function of brain, heart, and other organs.

In the foregoing examples in which a compound of the invention is used for *ex-vivo* applications, or to treat responsive cells such as neuronal tissue, retinal tissue, heart, lung, liver,

kidney, small intestine, adrenal cortex, adrenal medulla, capillary endothelial, testes, ovary, or endometrial cells or tissue, the invention provides a pharmaceutical composition in dosage unit form adapted for protection or enhancement of responsive cells, tissues, or organs distal to the vasculature which comprises, per dosage unit, an effective non-toxic amount within the range from about 0.01 pg to 5 mg, 1 pg to 5 mg, 500pg to 5 mg, 1 ng to 5 mg, 500 ng to 5 mg, 1 μg to 5 mg, 500 μg to 5mg, or 1 mg to 5 mg of compound of the invention and a pharmaceutically acceptable carrier. In a preferred embodiment, the amount of a compound of the invention is within the range from about 1 ng to 5mg.

In a further aspect of the invention, EPO administration was found to restore cognitive function in animals having undergone brain trauma. The compounds of the invention would be expected to have the same cellular protective effects as EPO. After a delay of either 5 days or 30 days, EPO was still able to restore function as compared to sham-treated animals, indicating the ability of a EPO to regenerate or restore brain activity. Thus, the invention is also directed to the use of a compound of the invention for the preparation of a pharmaceutical composition for the treatment of brain trauma and other cognitive dysfunctions, including treatment well after the injury (e.g. three days, five days, a week, a month, or longer). The invention is also directed to a method for the treatment of cognitive dysfunction following injury by administering an effective amount of a compound of the invention. Any compound of the invention as described herein may be used for this aspect of the invention.

Furthermore, this restorative aspect of the invention is directed to the use of any of the compounds of the invention herein for the preparation of a pharmaceutical composition for the restoration of cellular, tissue, or organ dysfunction, wherein treatment is initiated after, and well after, the initial insult responsible for the dysfunction. Moreover, treatment using a compound of the invention can span the course of the disease or condition during the acute phase as well as a chronic phase.

The compound of the invention, however is nonerythropoietic, i.e., it is capable of exerting the activities described herein without causing an increase in hemoglobin concentration or hematocrit. Such a non-erythropoietic compound is especially preferred in instances wherein the methods of the present invention are intended to be provided chronically. In another embodiment, a compound of the invention is given at a dose greater than that of a corresponding dose (W/W) of natural erythropoietin which would be necessary to maximally stimulate erythropoiesis. As noted above, a compound of the invention does not have erythropoietic activity, and therefore the above dosages expressed in units are merely exemplary for

corresponding amounts of natural erythropoietin; herein above molar equivalents for dosages are provided which are applicable to any compound of the invention.

Examples:

The present invention may be better understood by reference to the following non-limiting examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Example 1:

The starting material of the process in this example was purified recombinant human EPO.

First the protein concentration was adjusted by ultrafiltration for the purpose of keeping a low process volume. The protein concentration was adjusted to 3 mg/ml.
The ultrafiltration was performed by means of a BioMax (Millipore) with a MWCO of 5 kDa.

After completion of the concentration step, the EPO solution was mixed with 0.5 M potassium borate tetra hydrate 0.5 M potassium cyanate, at pH 9.0 the solution was incubated at 32°C for 24 hours.

The desalting of the reaction mixture of EPO and cyanate was performed by gelfiltration. The protein was desalted to a 25 mM Tris, 50 mM NaCl pH 8.5 buffer. A G-25 Fine (Amersham Biosciences) resin was employed.
Using a flow of 90 cm/h on an approximately 15 cm high column a sample load of approximately 20% of the column volume was applied.
The desalted carbamylated EPO was collected for further processing.

- At this point, the polymer/aggregate content was 7.3 %.

The next step was the removal of aggregates and polymers performed by a purification step using anionexchange. A SOURCE 30Q (Amersham Biosciences) resin was employed for the purification. Approximately 4.5 mg/ml of carbamylated EPO was applied to the column.

The running buffer A was: 25 mM Tris, 50 mM NaCl pH 8.5, and elution buffer B: 25 mM Tris, 1 M NaCl pH 8.5. The gradient was performed with 0-30 % over 20 column volumes the main peak of carbamylated EPO was collected and pooled.

The pool from the purification step was adjusted to a concentration > 0.5 mg/ml and buffer changed to a 20 mM citrate, 100 mM NaCl buffer using a dia/ultrafiltration tangential flow filtration unit.

The concentration and bufferchange was performed on a $0.1m^2$ BioMax (Millipore) with a MWCO of 5 kDa.

Finally the purified biopharmacutical drug substance was 0.22 µm filtrated using a Millipak filter (Millipore) to reduce contamination.

The process resulted in carbamylated EPO with properties making it useful as a biopharmaceutical;

- The polymer/aggregate content was 0.5 % as determined by SEC-HPLC
- The carbamylated lysines and N-terminal amine was 100% as determined by aminoacid analysis
- The concentration was > 0.5 mg/ml

Characterization using MALDI-TOF for the determination of the change in the intact mass both of PNGase treated protein and for the protein with the N-glycans was performed. In addition MALDI-TOF peptide mass fingerprint analysis/LC-MS/MS analysis was perfomed. The conclusion was that the 8 lysines and the N-terminal was carbamylated. No other carbamylated aminoacids were detected and no modifications of the glycans were detected.

Claims:

1. A method for producing a carbamylated erythropoietin protein having less than about 40% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein as measured by ESI-mass spectrometry, which method comprises contacting an amount of erythropoietin with an amount of cyanate at a temperature, pH, and for a time period sufficient for the amine groups on the lysines and the N-terminal amino acids of the erythropoietin to become 90% carbamylated.

2. The method of claim 1, wherein the carbamylated erythropoietin protein is human erythropoietin.

3. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 30% aggregated protein.

4. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 20% aggregated protein.

5. The method of claim 1, wherein the carbamylated erythropoeitin protein has less than about 10% aggregated protein.

6. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over- and under-carbamylated protein.

7. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over- and under-carbamylated protein.

8. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 10% by weight of over- and under-carbamylated protein.

9. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over-carbamylated protein.

10. The method of claim 1, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over-carbamylated protein.

11. The method of claim 1, wherein the carbamylated erythropoietin protein has less than

about 10% by weight of over-carbamylated protein.

12, The method of claim 1, wherein the concentration of erythropoietin protein contacted with the cyanate is from about 0.05 mg/ml to about 10 mg /ml.

13. The method of claim 1, wherein the concentration of erythropoietin protein contacted with the cyanate is about 2 mg/m1 to abou 5 mg/ml.

14. The method of claim 1, wherein the concentration of the cyanate is from about 0.05 M to about 10 M.

15. The method of claim 1, wherein the concentration of the cyanate is from about 0.05 M to about 2 M.

16. The method of claim 1, wherein the temperature ranges from about 0°C to about 60° C.

17. The method of claim 1, wherein the temperature ranges from about 30°C to about 34°C.

18 The method of claim 1, wherein the pH is from about 7 to about 11.

19. The method of claim 1, wherein the pH is from about 8 to about 10.

20. The method of claim 1, wherein the time period is from about 10 minutes to about 30 days.

21. The method of claim 1, wherein the time period is from about 1 hour to about 5 days.

22. The method of claim 1, wherein the erythropoietin protein is contacted with the cyanate in the presence of a buffer.

23. The method of claim 22, wherein the buffer is borate.

24. The method of claim 22, wherein the concentration of the buffer is from about

0.05 M to about 2 M.

25. The method of claim 22, wherein the concentration of the buffer is from about 0.1 M to about 1 M.

26. The method of claim 22, wherein the concentration of the buffer is about 0.5M.

27. The method of claim 1, wherein the concentration of the erythropoietin protein contacted with the cyanate is from about 0.05 mg/ml to about 10 mg/ml, the concentration of the cyanate is from about 0.05 M to about 10 M, the temperature ranges from about 0°C to about 60°C, the pH is from about 7 to about 11, and the time is from about 10 minutes to thirty days.

28. The method of claim 1, wherein the concentration of the erythropoietin protein contacted with the cyanate is from about 2 mg/ml to about 5 mg/ml, the concentration of the cyanate is from about 0.05 M to about 2 M, the temperature ranges from about 30°C to about 34°C, the pH is from about 8 to about 10, and the time is from about 1 hour to 5 days.

29. The method of claim 1, wherein the concentration of the erythropoietin protein contacted with the cyanate is about 3 mg/ml, the concentration of the cyanate is about 0.5 M, the temperature is about 32°C, the pH is about 9.0, and the time period is about 24 hours.

30. A method for producing a carbamylated erythropoietin protein having less than about 3% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein as measured by ESI-mass spectrometry, comprising purifying the carbamylated erythropoietin using anion exchange, cation exchange, hydrophobic interaction chromatography, reverse phase chromatography, affinity chromatography or size exclusion chromatography.

31. The method of claim 30, wherein the carbamylated erythropoietin protein is human erythropoietin.

32. The method of claim 30, wherein 90% of the amine residues on the lysines and the N-terminal amino acid of the erythropoietin are carbamylated.

33. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 2.5% aggregated protein.

34. The method of claim 30, wherein the carbamylated erythropoietin protein has about 0.5% aggregated protein.

35. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over- and under-carbamylated protein.

36. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over- and under-carbamylated protein.

37. The method of claim 30 wherein the carbamylated erythropoietin protein has less than about 10% by weight of over- and under-carbamylated protein.

38. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over-carbamylated protein.

39. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over-carbamylated protein.

40. The method of claim 30, wherein the carbamylated erythropoietin protein has less than about 10% by weight of over-carbamylated protein.

41. A method for producing a carbamylated erythropoietin protein having less than about 3% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein as measured by ESI mass spectrometry, which method comprises:

(a) contacting an amount of an erythropoietin protein with an amount of cyanate at a temperature, pH, and for a time period sufficient for 90% of the amine residues on the lysine and the N-terminal amino acids of the erythropoietin to become carbamylated; and

(b) purifying the carbamylated erythropoietin protein using anion exchange, cation exchange, hydrophobic interaction chromatography, reverse phase chromatography, affinity chromatography or size exclusion chromatography.

42. The method of claim 41, wherein the carbamylated erythropoietin protein is human erythropoietin.

43. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 2.5% aggregated protein.

44. The method of claim 41, wherein the carbamylated erythropoietin protein has about 0.5% aggregated protein.

45. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over- and under-carbamylated protein.

46. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over- and under-carbamylated protein.

47. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 10% by weight of over- and under-carbamylated protein.

48. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over-carbamylated protein.

49. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over-carbamylated protein.

50. The method of claim 41, wherein the carbamylated erythropoietin protein has less than about 10% by weight of over-carbamylated protein.

51. The method of claim 41, wherein the concentration of erythropoietin protein contacted with the cyanate is from about 0.05 mg/ml to about 10 mg/ml.

52. The method of claim 41, wherein the concentration of erythropoietin protein contacted with the cyanate is from about 2 mg/ml to about 5 mg/ml.

53. The method of claim 41, wherein the concentration of erythropoietin protein contacted with the cyanate is about 3 mg/ml.

54. The method of claim 41, wherein the concentration of the cyanate is from about 0.05 M to about 10 M.

55. The method of claim 41, wherein the concentration of the cyanate is from about 0.05 M to about 2 M.

56 The method of claim 41, wherein the concentration of the cyanate is about 0.5 M.

57. The method of claim 41, wherein the temperature ranges from about 0°C to about 60° C.

58. The method of claim 41, wherein the temperature ranges from about 30°C to about 34° C.

59. The method of claim 41, wherein the temperature is about 32°C.

60. The method of claim 41, wherein the pH is from about 7 to about 11.

61. The method of claim 41, wherein the pH is from about 8 to about 10.

62. The method of claim 41, wherein the pH is about 9.

63. The method of claim 41, wherein the time period is from about 10 minutes to about 30 days.

64. The method of claim 41, wherein the time period is from about 1 hour to about 5 days.

65. The method of claim 41, wherein the time period is about 24 hours,

66. The method of claim 41, wherein the erythropoietin protein is contacted with the cyanate in the presence of a buffer.

67, The method of claim 66, wherein the buffer is borate.

68. The method of claim 66, wherein the concentration of the buffer is from about 0.05 M to about 2 M.

69, The method of claim 66, wherein the concentration of the buffer is from about 0.1 M to about 1 M.

70, The method of claim 66, wherein the concentration of the buffer is about 0.5M.

71, The method of claim 41, wherein the concentration of erythropoietin protein

contacted with the cyanate is from about 0.05 mg/ml to about 10 mg/ml, the concentration of the cyanate is from about 0.05 M to about 10 M, the temperature ranges from about 0°C to about 60°C, the pH is from about 7 to about 11, and the time is from about 10 minutes to thirty days.

71. The method of claim 41, wherein the concentration of erythropoietin protein contacted with the cyanate is from about 2 mg/ml to about 5 mg/ml, the concentration of the cyanate is from about 0.05 M to about 2 M, the temperature ranges from about 30°C to about 34°C, the pH is from about 8 to about 10, and the time is from about 1 hour to 5 days.

72. The method of claim 34, wherein the concentration of the erythropoietin protein contacted with the cyanate is about 3 mg/ml, the concentration of the cyanate is about 0.5 M, the temperature is about 32°C, the pH is about 9.0, and the time period is about 24 hours.

74. A carbamylated erythropoietin protein having 90% carbamylation of the primary amines of the lysine and amino terminal amino acids, less than about 3% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein as measured by ESI-mass spectrometry.

75. The carbamylated erythropoietin protein of claim 74, wherein the erythropoietin protein is human erythropoietin,

76. The carbamylated erythropoietin protein of claim 74, having less than about 2.5% aggregated protein.

77. The carbamylated erythropoietin protein of claim 74, having about 0.5% aggregated protein.

78. The carbamylated erythropoietin protein of claim 74, wherein the amount of aggregated protein is measured by SEC-HPLC.

79. The carbamylated erythropoietin protein of claim 74, wherein the carbamylation of the lysine and amino terminal amino acids is measured by MALDI-TOF.

80, The carbamylated erythropoietin protein of claim 74, having less than about 30% by weight of over- and under-carbamylated protein,

81, The carbamylated erythropoietin protein of claim 74, having less than about 20% by

weight of over- and under-carbamylated protein.

82. The carbamylated erythropoietin protein of claim 74, having less than about 10% by weight of over- and under-carbamylated protein.

83. The carbamylated erythropoietin protein of claim 74, having less than about 30% of over-carbamylated protein.

84. The carbamylated erythropoietin protein of claim 74, having less than about 20% of over-carbamylated protein.

85. The carbamylated erythropoietin protein of claim 74, having less than about 10% of over-carbamylated protein.

86. A compound comprising a carbamylated erythropoietin protein having 90% carbamylation of the primary amines of the lysine and amino terminal amino acids, less than about 40% aggregated protein, and an amount of cyanate.

87. The compound of claim 86, wherein the carbamylated erythropoietin protein is human erythropoietin.

88. The compound of claim 86, wherein the carbamylated erythropoietin protein has less than about 30% aggregated protein,

89. The compound of claim 86 wherein the carbamylated erythropoietin has less than about 20% aggregated protein.

90. The compound of claim 86, wherein the carbamylated erythropoietin protein has less than about 15% aggregated protein.

91. The compound of claim 86, wherein the carbamylated erythropoietin protein has less than about 10% aggregated protein.

92. The compound of claim 86, wherein the carbamylated erythropoietin protein has less than about 7% aggregated protein.

93, The compound of claim 86, wherein the amount of aggregated protein is measured by SEC-

HPLC.

94. The compound of claim 86, wherein the carbamylation of the lysine and amino terminal amino acids is measured by MALDI-TOF.

95. A carbamylated erythropoietin protein having carbamylation of 90% of the primary amines of the lysine and amino terminal amino acids, less than about 3% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein as measured by ESI-mass spectrometry, which is the product of the process comprising the steps of

(a) contacting an amount of an erythropoietin protein with an amount of a cyanate at a temperature, pH, and for a time period sufficient for 90% of the amine residues on the lysines and the N-terminal amino acid of the erythropoietin protein to become carbamylated; and

(b) purifying the carbamylated erythropoietin protein using anion exchange, cation exchange, hydrophobic interaction chromatography, reverse phase chromatography, affinity chromatography or size exclusion chromatography.

96. The carbamylated erythropoietin protein of claim 95, wherein the erythropoietin protein is human erythropoietin.

97. The carbamylated erythropoietin protein of claim 95, having less than about 2.5% aggregated protein.

98. The carbamylated erythropoietin protein of claim 95, having about 0.5% aggregated protein.

99. The carbamylated erythropoietin protein of claim 95, wherein the amount of aggregated protein is measured by SEC-HPLC.

100. The carbamylated erythropoietin protein of claim 95, wherein the carbamylation of the lysine and amino terminal amino acids is measured by MALDI-TOF.

101. The carbamylated erythropoietin protein of claim 95, having less than about 30% by weight of over- and under-carbamylated protein,

102. The carbamylated erythropoietin protein of claim 95, having less than about 20% by weight of over- and under-carbamylated protein

103. The carbamylated erythropoietin protein of claim 95, having less than about 10% by weight of over- and under-carbamylated protein.

104. The carbamylated erythropoietin protein of claim 95, having less than about 20% by weight of over-carbamylated protein.

105. The carbamylated erythropoietin protein of claim 95, having less than about 10% by weight of over-carbamylated protein.

106. The carbamylated erythropoietin protein of claim 95, having less than about 5% by weight of over-carbamylated protein.

107. The carbamylated erythropoietin protein of claim 95, wherein the process comprises the concentration of the erythropoietin protein contacted with the cyanate is about 3 mg/ml, the concentration of the cyanate is about 0.5 M, the temperature is about 32°C, the pH is about 9.0, and the time period is about 24 hours.

108. A pharmaceutical composition comprising a therapeutically effective amount of a carbamylated erythropoietin protein having carbamylation of 90% of the primary amines of the lysine and amino terminal amino acids, less than about 3% aggregated protein and less than about 40% by weight of over- and under-carbamylated protein, and a pharmaceutically acceptable carrier.

109. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein is human erythropoietin.

110. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 2.5% aggregated protein.

111. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has about 0.5% aggregated protein.

112. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over- and under-carbamylated protein.

113. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over- and under-carbamylated protein.

114. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than 10% by weight of over- and under-carbamylated protein.

115. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 30% by weight of over-carbamylated protein.

116. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 20% by weight of over-carbamylated protein.

117. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 10% over-carbamylated protein.

118. The pharmaceutical composition of claim 108, wherein the carbamylated erythropoietin protein has less than about 5% over-carbamylated protein.

119. The pharmaceutical composition of claim 108, wherein the carrier is a diluent, an adjuvant, or an excipient.

120. A method of treating a chronic condition or disease, comprising administering the pharmaceutical composition of claim 108.

121. A method of treating a subchronic condition or disease, comprising administering the pharmaceutical composition of claim 108.

122. A method of treating an acute condition or disease, comprising administering the pharmaceutical composition of claim 108.

123. A method of treating a disease of the central nervous system or peripheral nervous system, comprising administering the pharmaceutical composition of claim 108.

124. The method of claim 123, wherein the disease is a stroke, an ischemic event, a spinal cord injury, a traumatic brain injury, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia, or chemotherapeutic induced neuropathy.

125. Use of a compound according to claim 74 for the production of a pharmaceutical

composition comprising said compound, for treatment of a chronic condition or disease.

126. Use of a compound according to claim 74 for the production of a pharmaceutical composition comprising said compound, for treatment of a subchronic condition or disease.

127. Use of a compound according to claim 74 for the production of a pharmaceutical composition comprising said compound, for treatment of an acute condition or disease.

128. Use of a compound according to claim 74 for the production of a pharmaceutical composition comprising said compound, for treatment of disease of the central nervous system or peripheral nervous system.

129. The use according to claim 128, wherein the disease is a stroke, an ischemic event, a spinal cord injury, a traumatic brain injury, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia, or chemotherapeutic induced neuropathy.

130. A method for the production of carbamylated EPO characterized by

a. having a low degree of polymer/aggregate formation after the carbamylation step, and <2.5% polymer/aggregate content after purification, and
b. further that the EPO is carbamylated only on lysines and N-terminal free amines, and that all lysines are carbamylated, and
c. that the amount of contaminating over or under carbamylated EPO constitutes less than 30 % w/w of the total carbamylated EPO.

131. A composition comprising EPO carbamylated on all lysines and on the N-terminal free amines, and on which no other amino acids can be detected by MALDI-TOF peptide fingerprinting as being carbamylated, and that comprise contaminating over or under carbamylated EPO to an extent that does not exceed 30 % of the total carbamylated EPO, and which comprise less than 2.5 % polymer/aggregate

132. A pharmaceutical composition comprising a therapeutically effective amount of the composition of claim 131.

133. The use of a composition according to claim 131 for the production of a pharmaceutical composition for the treatment of diseases of the CNS and/or the peripheral nervous system.

134. The use according to claim 133 where the disease is a neurodegenerative disease.

135. The use according to claim 133 where the disease is stroke or another ischemic event in the CNS, spinal cord injury, traumatic brain injury, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimers disease, schizophrenia, chemotherapy induced neuropathy.

136. A method for the treatment of a patient suffering from diseases of the CNS comprising administering to said patient a pharmaceutically effective amount of the composition according to claim 132.

137. A method for the treatment of a patient according to claim 136, wherein the disease is a neurodegenerative disease.

138. A method for the treatment of a patient according to claim 136, wherein the disease is stroke or another ischemic event in the CNS, spinal cord injury, traumatic brain injury, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimers disease, schizophrenia, chemotherapy induced neuropathy.